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## **Napsin-A, TTF-1, *EGFR* and *ALK* status determination in lung primary and metastatic mucin-producing adenocarcinomas**

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**Table:**

2;

**Figures:**

1

**Abstract**

Pulmonary mucin-producing adenocarcinomas may be indistinguishable on conventional histology from a metastasis, as TTF-1 expression often is lacking and KRAS mutations are widely present even in extra-pulmonary sites. Few data have been reported on the diagnostic role of napsin-A and EGFR and ALK gene alterations in this challenging differential diagnosis. Seventy-seven surgically resected cases, including 53 primary and 24 metastatic tumors from different sites, were evaluated for Napsin-A, TTF-1 and ALK by immunohistochemistry and for EGFR mutations by direct sequencing. Overall, napsin-A expression in primary lung mucin-producing adenocarcinomas was 36% (8% mucinous, 17% colloid, 87.5% solid, and 100% signet ring cell) and TTF-1 expression reached an overall figure of 42% (12.5% mucinous, 33% colloid, 87.5% solid, and 100% signet ring cell). Metastatic mucinous adenocarcinomas did not react with napsin-A or with TTF-1. All primary and metastatic tumors lacked EGFR mutations, while a single case of signet ring cell lung adenocarcinoma showed ALK expression and rearrangement at FISH analysis. Napsin-A has a lower sensitivity compared to TTF-1 in primary mucin-producing adenocarcinomas of the lung. However, both antibodies have an absolute specificity, being always negative in metastatic mucinous adenocarcinomas. EGFR mutations and ALK translocation or expression are exceedingly rare in mucin-producing adenocarcinomas of the lung, resulting unnecessary as diagnostic tool in this setting.

**Key words:** lung; mucinous; adenocarcinoma; metastasis; Napsin-A; TTF-1; immunohistochemistry, EGFR.

**Running head:** Napsin-A and EGFR in mucinous adenocarcinomas

## INTRODUCTION

In the lung, the differential diagnosis between primary and metastatic adenocarcinomas may be challenging, particularly when dealing with mucin-producing adenocarcinomas. In fact, the morphologic features of pulmonary (mucinous, colloid, signet-ring cell, solid with mucin) and extrapulmonary mucin-producing adenocarcinomas, particularly from pancreato-biliary tract, are frequently overlapping<sup>1</sup>. Immunohistochemical studies with some specific markers of lung primary, namely thyroid transcription factor-1 (TTF-1), surfactant apoproteins and cytokeratin (CK) 7, and of extrapulmonary origin, as caudal-related homeobox gene (CDX2) and CK20, are generally helpful<sup>2,3</sup>. Nevertheless, lack of pulmonary markers and aberrant expression of CDX2 and CK20 have been well demonstrated in some mucin-producing adenocarcinomas of the lung<sup>4-13</sup>. In addition, metastatic adenocarcinomas may grow along alveolar structures in a lepidic pattern, then closely mimicking primary lung adenocarcinomas<sup>1</sup>.

While TTF-1 is still considered the most specific marker in differentiating lung primary versus metastatic adenocarcinomas<sup>14</sup>, some disturbing staining have been reported in several extrapulmonary adenocarcinomas, mainly from gynaecologic tract<sup>15,16</sup>, and different specificity have been observed using different clones recognizing TTF-1.<sup>17</sup>

Napsin-A is a relatively novel marker of lung and renal primary, consisting of an aspartic proteinase homologous to polypeptide TA02 involved in the maturation of surfactant and expressed in normal type II pneumocytes and intra-alveolar macrophages because of surfactant phagocytosis.<sup>18-21</sup> To date, only Wu et al<sup>22</sup> have investigated the role of napsin-A in mucin-producing adenocarcinomas of the lung and extrapulmonary mucinous adenocarcinomas of different sites, reporting high specificity but low sensitivity of Napsin-A expression in primary mucin-producing adenocarcinomas of the lung.

In the current study, we compared the value of TTF-1 and napsin-A in a series of 77 surgically resected lung primary (53 cases) and metastatic (24 cases) mucin-producing

adenocarcinomas. Genetic alterations involving epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) have a critical value in predicting clinical response to targeted molecular therapies with specific inhibitors,<sup>23-25</sup> but are also strikingly restricted to lung adenocarcinomas. Thus, molecular investigation of EGFR mutations by direct sequencing method and demonstration of ALK aberrant expression have been tested in all tumors of our series as a possible alternative diagnostic tool in discriminating primary versus metastatic mucinous adenocarcinomas.

## MATERIALS AND METHODS

**Case series.** The files of the Operative Unit of Pathologic Anatomy of the Azienda Arcispedale S. Maria Nuova of Reggio Emilia, the University Hospital Policlinico of Modena and the Unit of Pathologic Anatomy of the University Hospital of Torino/Orbassano were searched for cases of pulmonary primary and metastatic mucin-producing adenocarcinomas diagnosed from 1991 to 2011. Overall, 53 primary lung and 24 metastatic tumors were collected. All the cases consisted of surgical specimens (19 wedge resections and 58 pulmonary lobectomies). Surgical samples were processed according to conventional protocol<sup>26</sup> and the tissue was routinely fixed in 10% buffered formalin and paraffin-embedded. The histologic classification was based on review of all hematoxylin and eosin-stained sections of the primary tumor (mean, 3 slides for each tumor; range, 2–6 slides). Slide review was performed by two pathologists (GR, AC) using a multi-headed microscope. Primary lung adenocarcinomas were classified according to the 2004 World Health Organization (WHO) criteria<sup>27</sup> and the International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS) classification.<sup>28</sup>

**Immunohistochemistry.** For immunohistochemical analysis, the following antibodies were used: TTF-1 (clone 8G7G3 / 1, 1:500 dilution; Ventana, Tucson, AZ, USA) and napsin-A (polyclonal; Ventana; prediluted) with an automated immunostainer (BenchMark; Ventana), as reported previously.<sup>29</sup> In each case, 4- $\mu$ m-thick sections obtained from a representative block were air-dried overnight at 37°C, then deparaffinized in xylene and rehydrated through a decreasing concentration of alcohol to water; 3-3-diaminobenzidine was used as the chromogene and Harris hematoxylin as the counterstain. Alveolar type II cells served as positive internal controls. Negative controls were included in each test by substituting the primary antibodies with nonimmune mouse IgG, at the same concentration as that of the relevant primary antibody. A tumor was considered positive if more than 10% of the neoplastic cells reacted, with a moderate (2+) or strong (3+) intensity, in the relevant subcellular localization (nuclear for TTF-1 and granular cytoplasmic for napsin-A). Clinical and radiologic data of patients were collected from pathologic reports, clinical charts, referring physicians, or directly from the patient's families.

**Molecular analysis.** Molecular analysis was performed by direct sequencing PCR as previously described.<sup>30</sup> Briefly, 5-micron thick sections obtained from a representative paraffin-embedded block were deparaffinized by xylene, and tumor DNA was extracted using a manual microdissection method. Microdissected tumor cells were subjected to proteinase K treatment in a digestion buffer (50mM Tris [pH 8.5], 1 mM EDTA, 0.5% Tween 20) and then incubated overnight at 37°C. PCR was performed in 10 $\mu$ L reaction mix containing 1.0 $\mu$ g DNA, 1 $\mu$ L of commercial PCR buffer (Applied Biosystem), 1.0-2.0mM MgCl<sub>2</sub>, 200 $\mu$ M of each dNTP, 25pmol of each primer, and 1.5 units of AmpliTaq gold polymerase (Applied Biosystem). PCR reaction was carried out on Uno II Thermoblock (Biometra, Gottingen, Germany). Initial denaturation at 94°C for 10 minutes was followed by 41 cycles, and a final extension step (7 minutes at 72°C). The cycles included

denaturation at 95°C for 1 minute, annealing at 55-65°C for 1 minute, and extension at 72°C for 2 minutes. The amplified DNA was electrophoresed on 2% agarose gel for 1 hour at 110V. The amplification products were then purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions and were sequenced in both directions on an ABIPrism 310 automatic sequencer (Applied Biosystems) using the Big Dye Terminator Kit. The data were analyzed with the *Sequencing Analysis 5.2* Software (Applied Biosystem). The forward and reverse sequences of EGFR exons 18, 19, and 21 were analyzed.

The presence of ALK alteration was preliminarily screened using the following 2 antibodies against ALK: clone ALK1 (Ventana, prediluted) and clone 5A4 (Novocastra, 1:50 dilution). In case of any expression of ALK (from 1+, weak to 3+, strong) further confirmation of ALK rearrangement was performed by fluorescent in situ hybridization (FISH) using dual color break-apart probes (Vysis, Abbott Molecular) according to conventional recommendations. A positive case was recorded when rearrangement for ALK was present in more than 15% of tumor cells considering broken-apart green/red signals and/or single red signals.

**Statistical analysis.** Results were tested for significance using the Pearson's chi-square test. Differences were considered significant at  $P$  values < 0.05.

## RESULTS

Immunohistochemical results comparing the expression of napsin-A and TTF-1 are summarized in **Table 1**. Napsin-A was expressed in 19 out of 53 mucin-producing adenocarcinomas of the lung (36%), including 2 out of 25 (8%) mucinous adenocarcinomas (formerly mucinous bronchioloalveolar carcinomas), all 8 adenocarcinomas with signet ring features, 2 out of 12 (17%) colloid and 7 out of 8 (87.5%) solid-type adenocarcinomas. Strong (3+) expression for napsin-A was mainly

observed in signet ring and solid-type adenocarcinomas, while a moderate (2+) staining characterized colloid and mucinous type adenocarcinomas (**Figure 1**).

TTF-1 expression was observed in 22 out of 53 (41.5%) mucin-producing adenocarcinomas of the lung, in particular 3 out of 25 (12%) mucinous adenocarcinomas, all the 8 adenocarcinomas with signet ring features, 4 out of 12 (33%) colloid and 7 out of 8 (87.5%) solid-type adenocarcinomas.

Comparison of napsin-A and TTF-1 expression did not reveal discrepant results, but 3 napsin-A negative cases (1 mucinous and 2 colloid) were immunoreactive for TTF-1. No immunoreactivity for Napsin-A or TTF-1 was recorded in metastatic mucinous adenocarcinomas from colon, pancreas, breast, ovary and sinonasal tract.

No EGFR mutations were disclosed in primary and metastatic tumors, while only 1 case of pulmonary adenocarcinoma featuring signet ring cells expressed ALK protein and showed ALK rearrangement upon FISH testing (**Table 2**).

No significant differences were observed when comparing immunohistochemical results for napsin-A and TTF-1 both in the primary pulmonary mucin-producing adenocarcinomas and metastases.

## DISCUSSION

In the lung, histologic discrimination between primary and metastatic mucin-producing adenocarcinomas may be a formidable challenge on conventional stains, often requiring the use of immunomarkers. While the low sensitivity limits the role of TTF-1 in this differential diagnosis and the expression of intestinal differentiation markers (i.e., CK20 and CDX2) is not an uncommon occurrence, only few data have been reported on the use of napsin-A or specific gene alterations of lung adenocarcinomas, namely EGFR mutations and ALK rearrangement, in this peculiar setting of differential diagnosis.



Napsin-A is an aspartic proteinase acting in the maturation of surfactant recently and resulting a quite specific marker of adenocarcinomas taking origin from kidney and lung.<sup>31</sup> In primary pulmonary tumors, napsin-A is strictly expressed in 60% to 90% of adenocarcinomas, but never in squamous cell carcinomas and neuroendocrine tumors.<sup>32</sup> A weak staining for napsin-A has been observed only in occasional tumors outside the lung and kidney.<sup>33</sup> The specificity and sensitivity of napsin-A in discriminating adenocarcinoma lineage from other histotypes of non-small cell lung cancer (NSCLC) is considered akin to or slightly superior than that observed for TTF-1.<sup>31,32</sup> Among pulmonary adenocarcinomas, mucin-producing variants have demonstrated the lowest rate of TTF-1 expression, then limiting the value of TTF-1 in discriminating primary pulmonary from metastatic mucinous adenocarcinomas.<sup>5,6,9,11,32</sup> Napsin-A immunoreactivity in mucin-producing adenocarcinomas of the lung and other primary extrapulmonary mucinous adenocarcinomas (colorectum, breast, stomach, pancreas and ovary) has been recently analyzed by Wu et al.<sup>22</sup> The authors found an overall sensitivity of 33% and 42% in primary lung mucin-producing adenocarcinomas for napsin-A and TTF-1, respectively. Some discrepancies between the two markers have been noted with an higher rate of TTF-1 expression in solid, colloid and signet ring cell type.<sup>22</sup> Other sporadic studies reported a weak immunoreactivity of napsin-A in mucinous adenocarcinoma (formerly bronchioloalveolar carcinoma, mucinous type) ranging from 0% to 86%.<sup>34,35</sup> The results in the current study do not significantly differ from those reported by Wu et al<sup>22</sup> with an overall higher rate of TTF-1 (42%) than napsin-A (36%) expression in mucin-producing adenocarcinomas of the lung. As highlighted previously<sup>22</sup> discrepancies among different studies on this issue may be related to methodologies employed in performing immunostaining with different clones of napsin-A. In fact, Mukhopadhyay and Katzenstein<sup>36</sup> recently demonstrated that monoclonal antibody recognizing napsin-A is

less sensitive, but more specific than polyclonal antiserum in discriminating lung origin of adenocarcinomas.

In general, the low expression for napsin-A, as well as TTF-1, in some types (mucinous and colloid) of primary pulmonary mucin-producing adenocarcinomas limits their value in the distinction from metastatic mucin-producing adenocarcinomas. However, their specificity is 100% since all mucinous adenocarcinomas from other sites are entirely negative, both in the current paper and Wu's et al work.<sup>22</sup>

Theoretically, also molecular investigations could have a role in this differential diagnosis. While identical KRAS mutations are widely identified in pulmonary and extrapulmonary mucin-producing adenocarcinomas,<sup>37</sup> gene alterations involving EGFR and ALK seem to be much more restricted to primary adenocarcinomas of the lung.<sup>38</sup> Whether previous studies<sup>30,39-41</sup> have demonstrated that mucinous adenocarcinomas of the lung tend to be wild-type at EGFR mutational analysis, herein we observed that also the other variants of primary lung and metastatic mucin-producing adenocarcinomas do not harbor EGFR mutations. Several works have underlined the significant relationship between ALK rearrangement and signet ring cell pattern of lung adenocarcinomas,<sup>42-44</sup> but no studies have explored ALK alterations along the spectrum of mucin-producing pulmonary adenocarcinomas and metastatic mucinous adenocarcinomas of different origin. In light of the significant correlation between immunohistochemistry and FISH methods in detecting ALK rearrangement,<sup>45-47</sup> we originally screened all 77 cases with two different clones (ALK1 and 5A4) directed against ALK protein. Only one positive case (a pulmonary adenocarcinoma with signet ring cells) was detected, subsequently confirmed to harbor ALK rearrangement upon FISH testing. In this study, EGFR and ALK gene alterations were not detected in any case of metastatic mucinous extrapulmonary tumors.

The low frequency of napsin-A (8%) and TTF-1 (12%) expression and the lack of alterations of genes specifically related to lung adenocarcinomas further confirm the

extreme difficulty in discriminating primary mucinous adenocarcinomas of the lung from metastatic mucinous adenocarcinomas, once clinicoradiologic and morphology have failed. Nevertheless, in other types of mucin-producing lung adenocarcinomas, namely colloid, signet ring cell and solid types, expression of napsin-A specifically identified the lung as the primary site of 61% cases, a figure overlapping to that observed with TTF-1 (68%).

Despite in our series napsin-A did not add further information in TTF-1 negative cases, as reported by Wu et al<sup>22</sup> and Ye et al,<sup>48</sup> the coordinated use of TTF-1 and Napsin-A in mucin-producing adenocarcinomas of the lung could cover a larger spectrum of tumors, somehow obviating to the low sensitivity when employing a single marker.

In summary, mucin-producing adenocarcinomas of the lung represent several distinct tumor entities with different expression of napsin-A and TTF-1, possibly related to different derivation from lung structures.<sup>49</sup> Napsin-A immunoreactivity is very limited in mucinous adenocarcinoma (8%), but is 17%, 87.5% and 100% in colloid, solid and signet ring cell types, respectively. No expression has been noted for both napsin-A and TTF-1 in metastatic mucinous adenocarcinoma from colon, pancreas, ovary, breast and sinonasal tract. Finally, genetic alterations of EGFR and ALK have no diagnostic role in discriminating pulmonary from extra-pulmonary metastatic mucin-producing adenocarcinomas, apart from occasional ALK-rearranged adenocarcinomas composed of signet ring cells.

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## FIGURE LEGENDS

**Figure 1.** Immunoreactivity for Napsin-A in mucinous (A), colloid (B), signet ring cell (C) and solid (D) types adenocarcinomas of the lung.

## ABBREVIATIONS:

TTF-1: thyroid transcription factor-1

ALK: anaplastic lymphoma kinase

Napsin-A: novel aspartic proteinase of the pepsin family A

EGFR: epidermal growth factor receptor

KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

CDX2: caudal-related homeobox gene

**Table 1. Distribution of Napsin A and TTF-1 expression among lung primary and metastatic mucin-producing adenocarcinomas**

	Napsin A n/tot (%)	TTF-1
Lung primary adenocarcinoma (n=53)		
- Mucinous (n=25)	2/25 (8)	3/25 (12)
- Signet ring (n=8)	8/8 (100)	8/8 (100)
- Colloid (n=12)	2/12 (17)	4/12 (33)
- Solid (n=8)	7/8 (87.5)	7/8 (87.5)
<i>Overall</i>	19/53 (36)	22/53 (41.5)
Lung metastatic mucinous adenocarcinoma (n=24)		
- Pancreas (n=6)	0/6	0/6
- Ovary (n= 3)	0/3	0/3
- Breast (n=2)	0/2	0/2
- Colon (n=11)	0/11	0/11
- Sinonasal tract (n=2)	0/2	0/2
<i>Overall</i>	0/24	0/24

**Abbreviations:** TTF-1, thyroid transcription factor-1

**Table 2. Distribution of EGFR and ALK alterations among lung primary and metastatic mucin-producing adenocarcinomas**

	EGFR	ALK
	n/tot (%)	
Lung primary adenocarcinoma (n=53)		
- Mucinous (n=25)	0/25	0/25
- Signet ring (n=8)	0/8	1/8 (12.5)
- Colloid (n=12)	0/12	0/12
- Solid (n=8)	0/8	0/8
<i>Overall</i>	<i>0/53</i>	<i>1/53 (1.8)</i>
Lung metastatic mucinous adenocarcinoma (n=24)		
- Pancreas (n=6)	0/6	0/6
- Ovary (n= 3)	0/3	0/3
- Breast (n=2)	0/3	0/2
- Colon (n=11)	0/11	0/11
- Sinonasal tract (n=2)	0/2	0/2
<i>Overall</i>	<i>0/24</i>	<i>0/24</i>

**Abbreviations:** EGFR, Epidermal Growth Factor Receptor; ALK, anaplastic lymphoma kinase

Figure 1

